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13. ABSTRACT *(Maximum 200 words)*

This is the final report of our exploratory case-control study on breast cancer. This study aimed to examine whether risk factor profiles differ according to estrogen receptor (ER) status among African-American women. During the period of the project, we established collaborations with the Tennessee Cancer Reporting System, hospitals in the study areas, and basic science researchers; we formulated a series of data collection and quality-control procedures; we identified and interviewed a number of cases and controls that is nearly equivalent to that expected in the study design; we collected breast cancer tissue specimens and measured ER status for most cases; and we conducted some preliminary analyses with results that are presented in this report. We also have published a hypothesis article based on the molecular biological progress on the topic. At the time of data analysis for this report, we were still waiting for responses from some cases and their doctors. We still need to collect tumor tissues and measure ER status for some cases and recruit some controls because these procedures lag the interviews with cases. These can be done soon and more deliberated analyses will be conducted.

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Kangmin Zhu 3/30/99

PI - Signature Date

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INTRODUCTION

This is the final report of our case-control study on risk factors of breast cancer according to estrogen receptor (ER) status among African-American women ages 20-64.

Since the research team was able to be fully on staff in May 1997, we have identified and interviewed 201 breast cancer cases and 190 controls, nearly having reached the expected numbers of cases and controls proposed in the study design. According to the modification we suggested in our first annual report, we added the collection of tumor tissue specimens and the measurement of ER status (instead of medical record review) to the study. We have finished the collection for 176 cases and measurement for 158 cases at the time of data analysis for this report. Because we are still waiting for responses from some cases and their doctors and because tumor tissue collection/processing/measurement and the recruitment of controls lag the interviews with cases, data have not been complete at this point. The following section of the report is based on cases and controls for whom both interview and laboratory data were available. It is based on very preliminary analyses and is written in a very limited time in a format for a scientific publication. As soon as all data are collected, a manuscript with more thorough data processing and analysis will be written for publication.

BODY

INTRODUCTION

Breast cancer has two subgroups according to estrogen receptor (ER) status: ER-positive and ER-negative. Estrogen-related factors, such as nulliparity, age at first full-term pregnancy, age at menarche, and age at menopause, are known to be risk factors for breast cancer [Berstein and Ross, 1993]. Because estrogen executes its influence on the biological activity and growth rate of breast cells through hormone receptors [Rayter, 1991], whether these factors can increase the risk of breast cancer may depend upon the existence of estrogen receptors. Therefore, it is reasonable to hypothesize that risk factor profiles may differ according to ER status of tumor.

There have been a number of epidemiological studies assessing if breast tumors with different ER statuses vary in risk factors [Hildreth, et al, 1983, Hislop et al, 1986, McTiernan et al, 1986, Stanford et al, 1987, Cooper et al, 1989, Kreiger et al, 1991, Yoo et al, 1993, Nasca et al, 1994, Kushi et al, 1995, Potter et al, 1995]. These studies have examined risk factors including family history of breast cancer, parity, benign breast disease, age at

first birth, breast feeding, age at menarche, menopausal status, exogenous estrogen, body size, alcohol consumption and dietary factors. However, the results have varied, although a relative consistency could be found for family history of breast cancer, history of benign breast disease and parity (Zhu et al, 1997). Furthermore, no studies have targeted African-American women who are more likely to have ER-negative tumors that have a worse prognosis. This study aimed to examine if risk factor profiles are different between ER-negative and ER-positive cancers among African-American women.

MATERIALS AND METHODS

Study Subjects

This study used a case-control design. Cases consisted of African-American female patients diagnosed with breast cancer during 1995-97 and who lived in Davidson, Shelby and Hamilton counties, Tennessee. All breast cancer patients were histologically confirmed (ICD-O site code C50) [Percy et al, 1990]. Controls were comprised of African-American women without breast cancer who were selected through random-digit telephone dialing and frequency matched to cases by 5-year age range and county.

Cases were selected through the Tennessee Cancer Reporting System (TCRS). TCRS was established in 1986 and requires hospitals to report cancer patients within 6 months of their diagnosis. TCRS provided us with a list of eligible patients, their mailing addresses, their doctors and their doctors' addresses and telephone numbers. We sent a letter to the doctors for their consent to contact their patients. The letter we sent described the study and asked if we could contact their patients. If a physician did not return the consent form after two mails, one of our staff members called the physician's office to determine the status of the letter and faxed or mailed another copy of the letter and consent form when needed.

Patients with a doctor's consent were sent a cover letter and a consent form for their participation in the study. The letter introduced the study procedures and a woman's right as a study participant and asked if they would participate in the study. The second packet was mailed to those who did not respond to the first one. A reminder call (where a telephone was available) was made to women who did not reply to both mailings. For women who did not respond and did not have a telephone listed, we sent a nurse, a breast cancer survivor, a social worker or a research assistant with African-American ethnicity to their homes

to seek their consent. Only patients who completed a consent form were recruited as cases for the study.

We selected controls using random digit dialing techniques [Harlow and Davis, 1988]. We first grouped cases diagnosed in the same calendar year whose telephone area codes serve the same county, and then formed the sampling frame by age distribution of the cases in the area. By randomly selecting one of the telephone prefixes of the cases and adding the last four random-selected digits, a call was made to find an eligible woman according to ethnic background and age range.

For each telephone number called, interviewers determined (1) whether it was a residential or nonresidential (business line, cellular network, fax machine, disconnected, or changed to another number,) number; (2) whether there were any eligible women for a residential number; (3) how many eligible women there were (randomly select one, if more than one eligible women); and (4) whether an eligible woman consented to have an interview. Up to 9 calls over a two-week period, including 3 day-time, 3 evening, and 3 weekend calls, were made for a telephone number that was not answered. If an eligible woman was identified, we described the study purposes and procedures, and asked whether she would accept a telephone interview. For a woman who agreed to participate, a

telephone interview was conducted.

To achieve a high response rate, we used a monetary incentive for both cases and controls ($25 for a completed interview and a drawing for an award of $200). We also provided cases with $10 for their agreement to release their tumor tissue specimens.

TCRS provided us 617 eligible patients with breast cancer. Out of the patients, 118 had no doctors identified. Out of the remaining 295 doctors available, 226 has responded to the study. The number of cases with a doctor's consent has been 305, out of which 203 participated in the study. These are not final statistics since the process of getting consent is still underway for some doctors and cases.

We identified 231 women eligible as frequency-matched controls. Out of the women, 190 had an interview (82.3%).

Collection of Epidemiological Data

We used telephone interview technique for the information on breast cancer risk factors. Telephone interviewers were trained on conversation skills on the telephone, ways to address the concerns a subject may have, and how to conduct an interview on the

questionnaire. They were also trained to improve their performance in reducing under-reporting of information and item non-response, avoiding inductive questioning and evading inferring from an incomplete or inadequate reply. They were asked to examine completed questionnaires immediately after an interview for any errors, inconsistencies, unusual answers and missing values, and to make corrections or compensations where possible. An overview of interview procedures and a brief interview guide were provided to the interviewers.

Information collected included demographic variables, reproductive and menstrual history, medical history, family history of cancer, personal habits (smoking, alcohol consumption and exercise), and anthropometric variables (weight and height).

Because study women might not be able to recall the use of oral contraceptive pills accurately, we also sent them a set of colored OC pill pictures and a short form with a paycheck after the telephone interview. The women were asked to complete the OC form and return it to us using the enclosed stamped envelope.

Tumor tissue collection and ER measurement

Paraffin-embedded tumor tissue samples were collected from

hospitals where cases were pathologically diagnosed. Tissue slides were made as soon as the samples were available. For a few cases whose tumor tissues were not available, their pathological reports were reviewed for the determination of estrogen receptor status (n=19 at the time of data analysis for this report).

ER status was measured using the immunohistochemical method [Chaudhuri et al, 1993, Thorpe 1988, Ferno et al, 1996]. This method uses monoclonal antibodies directed against ER to detect the existence of Ers and can be used to paraffine-embedded tumor tissue specimens.

For ER staining, we heated paraffin sections of a tissue slide, deparaffinized and hydrated it. Antigen was retrieved and ER antibody was then applied, following a number of steps. After ER staining was completed, ER positive cells were assessed under a light microscope. ER-positiveness was defined as 5% or more neoplastic cell nucei showing staining with ER monoclonal antibody [Chaudhuri et al, 1993].

Data Analysis

As the first step of data analysis, we described the

distribution of demographic characteristics for three comparison groups: ER-positive, ER-negative and controls. Then, polytomous logistic regression is used to identify risk factors for breast cancer according to ER status (Dubin and Pasternack, 1984, Hosmer and Lemeshow, 1989). Using this method, two case groups with different ER statuses and a control group can be compared. In contrast with pairwise logistic regressions by ER status, polytomous logistic regression have slight improvement in estimator precision and can simultaneously test whether odds ratios (ORs) from the two case-control comparisons are all one and whether ORs from several case-control comparisons are homogenous even if confounders are different for the comparisons. In the analysis, we always put in the models demographic variables such as age, marital status, educational level, and annual family income. Other factors are selected into the model if the confidence interval of an odds ratio (OR) estimate for either or both of the case subgroups excludes the unity. A forward approach is used, with which a variable with an OR confidence interval excluding one and with most significant likelihood test enters the model at each step. At the time of writing this report, we only did the preliminary analyses, in which we used pairwise logistic regression for ER- and ER+ tumors, respectively. The odd ratio for a risk factor and its 95% confidence interval (CI) were estimated while only controlling for demographic variables.

RESULTS

Table 1 shows demographic characteristics of study subjects. Cases with ER+ tumors tended to be older, compared with those with ER- breast cancer and controls. They were also more likely to be married. However, ER- cases were more likely to have ever been employed, relative to ER+ cases and controls. Compared with controls, cases seemed to have higher educational level (some college or above) and household income.

These demographic variables were adjusted when assessing the relationship between a potential risk factor and breast cancer. Table 2 presents the odds ratio estimates of menstrual, reproductive and contraceptive factors. The odds ratio estimates were close to the unity and did not differ between ER- and ER+ tumors for age at menarche, number of days between two menstrual periods, age at 1st pregnancy, and use of birth control pills. Compared with having ever had one pregnancy, women with more pregnancies seemed to have lower risk of developing breast cancer for both types. However, postmenopausal status and having being ever pregnant seemed inversely associated with the disease with ER- status only. Increasing number of days of period tended to increase the risk for ER+ cancer only.

The use of estrogen or progesterone and history of surgery on ovaries were not associated with either ER- or ER+ tumors (Table 3). History of radioactive therapy tended to be related to the increased risk of both types of the disease. However, history of benign breast disease was more likely to be associated with ER- tumors and history of other cancers was more likely to be related to ER+ disease.

Family history of breast cancer was associated with increased risk of both ER- and ER+ tumors, with a higher odds ratio estimate for history of the disease in second degree relatives (table 4). The association with family history seemed to be stronger for ER+ cancer.

Cigarette smoking was not related to both ER- and ER+ tumors, while alcohol consumption seemed inversely associated with them (table 5). Increased risk with older age at first sex or first sex on a regular basis was shown for both types of breast cancer although dose-effect relation was not found for age at the first regular sex. The risk of breast cancer associated with electric blanket use seemed to be slightly increased for ER- tumors.

DISCUSSION

Although we have nearly completed data collection for the project, some remaining subjects need to be identified and interviewed and ER status of the tumor for some cases needs to be measured. As a result, only very preliminary analyses have been done and any results from the analyses are premature. Therefore, we hereby have only a brief discussion based on the preliminary results on the most important factors.

Our preliminary results showed an association between family history of breast cancer and the disease despite its ER status. This has been shown in four previous studies in which relative risks of family history associated with breast cancer were similar between ER-positive and ER-negative tumors (Hislop et al, 1986, McTierman et al, 1986, Stanford et al, 1987, Cooper et al, 1989). However, two other studies showed that family history is only related to ER-negative cancer (Yoo et al, 1993) or has a stronger association with estrogen receptor-negative tumors (5.7 for ER-negative tumors vs 1.8 for ER-positive tumors) (Kreiger et al, 1991).

Unlike most of previous studies (Hildreth et al, 1983, Hislop et al, 1983, McTiernan et al, 1986, Stanford et al, 1987, Cooper et al, 1989, Kreiger et al, 1991, Yoo et al, 1993), our study

found that having never been pregnant might increase the risk of ER- breast cancer. The previous studies showed that the tendency that nulliparous women are more likely to develop breast cancer was shown only for estrogen receptor-positive cancers. However, the relative risks in both our study and these previous studies were not significantly different from the unity in general.

Previous studies on age at menarche have varied in results. Late age at menarche tended to be related to decreased risk for both ER-positive and ER-negative tumors in one study (Kreiger et al, 1991) while another study found this tendency only for ER-negative breast cancers (Cooper et al, 1989). On the contrary, the increased risk associated with late age at menarche was demonstrated for both statuses of the steroid receptor in the study by McTiernan et al (McTiernan et al, 1986). Differing from these studies, our study did not show an association between age at menarche and either type of breast cancer.

Only two studies assessed the relationship between menopausal status and breast cancer according to ER status (Stanford et al, 1987, Kreiger et al, 1991). The studies found no differences in relative risk between ER-positive and ER-negative tumors, after adjusting for age. However, our study showed that the OR estimates were 2.0 (95% CI 1.0-3.9) and 0.5 (95%CI 0.2-1.3) for

ER- and ER+ tumors, respectively, suggesting a possible difference.

Most previous studies on benign breast disease have observed a tendency that women with the benign disease are at higher risk to develop both ER-positive and ER-negative breast cancers (Hislop et al, 1986, McTiernan et al, 1986, Stanford et al, 1987, Kreiger et al, 1991). However, with the exception of Kreiger et al's study in which relative risks were significantly higher than one for both receptor statuses (Kreiger et al, 1991), increased relative risk was significant only for one ER status: ER-positive tumors (Hislop et al, 1986, Stanford et al, 1987) or ER-negative disease (McTernan et al, 1986). In contrast, the risk associated with benign breast disease tended to be lower for ER-negative cancers and higher for ER-positive tumors in some other studies (Hildreth et al, 1983, Cooper et al, 1989). Although the OR estimates were not significantly different from one for both types of breast cancer in our study, the risk of benign breast disease tended to be increased for ER- tumors.

Our results on the use of estrogen agreed with those from previous studies (Hildreth et al, 1983, McTiernan et al, 1986, Stanford et al, 1987, Cooper et al, 1989): no significantly increased relative risks could be observed for the estrogen-

use/breast-cancer association for either ER- or ER+ tumors.

Previous studies on smoking in relation to breast cancer risk in terms of ER status have been inconsistent. While two studies did not observe the association of smoking with either ER-positive or ER-negative tumor (McTiernan et al, 1986, Stanford et al, 1987), one study found the association with ER-negative cancers only (Cooper et al, 1989) and the other found the association with ER-positive tumors only (London et al, 1989). Our study did not find an association between smoking and either type of breast cancer.

Our study showed an inverse relation between alcohol consumption and breast cancer and such an association did not vary depending on ER status. Some of the previous studies also did not find the difference between ER- and ER+ tumors on the relationship between alcohol drinking and breast cancer (McTiernan et al, 1986, Cooper et al, 1989). However, McTiernan et al. found that women who drank 7 drinks or more per week had an elevated risk (for both types of the disease) (McTiernan et al, 1986). In some other studies, the possible differences between the two types of breast cancer were shown with a finding that alcohol was only related to ER+ tumors (Nasca et al, 1994) or ER- tumors (Potter et al, 1995).

Two hypotheses have been raised about the role of estrogen receptor status in the development of breast cancer (Habel and Stanford, 1993, Harlan et al, 1993). One hypothesis is that ER status may represent different stages in the disease progress. The other hypothesis considers ER-positive and ER-negative cancers as different entities that may have somewhat different risk factor profiles. This study showed some possible differences in some factors between ER- and ER+ tumors. However, the confidence intervals of odds ratio estimates from the two types of the disease were usually overlapped, preventing us from a clear conclusion that ER- and ER+ cancers have somewhat different risk factor profiles. Our results from African-American women differed to some extent from those from the previous studies, suggesting a need of more studies in the population. Again, our analyses presented in this report were very preliminary. More deliberated analyses will be done when data are entirely complete.

CONCLUSIONS

With an extension of six months, we have successfully conducted the research project. During the period of the project, we established collaborations with the Tennessee Cancer Reporting System, hospitals in three counties, and basic science researchers; we formulated a series of data collection and

quality-control procedures; we identified and interviewed approximately an expected number of study subjects; we collected tumor tissue samples and measured ER status; we conducted some preliminary analyses; and we have already published a related article. Population-based epidemiological studies have been rare in Tennessee. Therefore, we had to face to some innate problems such as less-research-oriented cancer registry and barriers to obtaining doctors' consent. It has been well known that African-Americans are less likely to participate in a study. We had to make much more effort to recruit study subjects (such as visiting women in three counties to increase the participation rate), compared with studies in other populations. In addition, we had some other adverse conditions: a late start of the project due to a very long position-control and hiring process within the school, inadequate funding and research staff vs. additional research activities such as home visits, and the absence of the research assistants (resignation or maternity leave) during the last several months. Considering these difficulties, our research team has been successful in reaching the goal indicated in the proposal. Especially, our project coordinator, Mrs. Sandra Hunter, has done a terrific job. Because no studies on the topic have been done in African-American women who are more likely to develop ER-negative tumors that have a worse prognosis, any results from our study will provide evidence on whether ER-

negative and ER-positive tumors differ in risk factors in African-American women. The infrastructure, network and collaborations established during the project and the data we have obtained have laid a solid fundamental for the development of more research projects. We deeply appreciate the Department of Defense Breast Cancer Research Program for the support of this study and appreciate the technical reviewers of the proposal and annual reports for their comments.

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APPENDICES

1. Table 1. Demographic characteristics of study subjects
2. Table 2. Odds ratio estimates of menstrual, reproductive or contraceptive factors
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4. Table 4. Odds ratio estimates of family history of breast cancer
5. Table 5. Odds ratio estimates of personal habits or behavior
6. Published article

Table 1. Demographic characteristics of study subjects, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1997

Variable		Controls	ER- cases	ER+ cases
Age at reference date	20-39	22 (11.6%)	13 (13.1%)	6 (7.7%)
	40-44	34 (17.9%)	17 (17.2%)	12 (15.4%)
	45-49	43 (22.6%)	23 (23.2%)	19 (24.4%)
	50-54	41 (21.6%)	21 (21.2%)	13 (16.7%)
	55-59	24 (12.6%)	13 (13.1%)	12 (15.4%)
	60-65	26 (13.7%)	12 (12.1%)	16 (20.5%)
Marital status at reference date	Married	80 (42.6)	43 (43.4%)	39 (50.0%)
	Separated	22 (11.7%)	14 (14.1%)	5 (6.4%)
	Divorced	35 (18.6%)	22 (22.2%)	19 (24.4%)
	Widowed	23 (12.2%)	10 (10.1%)	5 (6.4%)
	Never Married	28 (14.9%)	10 (10.1%)	10 (12.8)
Employment at reference date	No	66 (34.9%)	24 (24.2%)	26 (33.3%)
	Yes	123 (65.1%)	75 (75.8%)	52 (66.7%)
Education Level	Elementary school	1 (0.5%)	0 (0.0%)	0 (0.0%)
	Middle School	5 (2.6%)	6 (6.1%)	2 (2.6%)
	High school	75 (39.7%)	31 (31.3%)	20 (25.6%)
	Vocational school	23(12.2%)	8 (8.1%)	13 (16.7%)
	Some college	47 (24.9%)	32 (32.3%)	18 (23.1%)
	College	17 (9.0%)	15 (15.2%)	12 (15.4%)
	Graduate or profe-Ssional school	18 (9.5%)	7 (7.1%)	12 (15.4%)

Table 1 continued

Variable		Controls	ER- cases	ER+ cases
Religion	None	3 (1.6%)	3 (3.0%)	0 (0.0%)
	Protestant	149 (78.4%)	75 (75.8%)	54 (69.2%)
	Catholic	3 (1.6%)	3 (3.0%)	6 (7.7%)
	Other	33 (17.4%)	18 (18.2%)	18 (23.1%)
Household Income (dollars)	<15,000	63 (34.4%)	26 (27.7%)	20 (26.7%)
	15,000-29,999	53 (29.0%)	20 (21.3%)	12 (16.0%)
	30,000-44,999	38 (20.8%)	23 (24.5%)	21 (28.0%)
	45,000-59,999	15 (8.2%)	11 (11.7%)	12 (16.0%)
	>=60,000	11 (6.0%)	14 (14.9%)	9 (12.0%)

Table 2. Odds ratio estimates of menstrual, reproductive or contraceptive factors for breast cancer by ER status, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1997

Factor		ER- cases		ER+ cases	
		OR*	95%CI**	OR	95%CI
Age at menarche	<=12	1.0		1.0	
	>12	0.8	0.4-1.5	1.1	0.6-2.2
Menstrual status	No	1.0		1.0	
	Yes	2.0	1.0-3.9	0.5	0.2-1.3
# of days between two menstrual period	<28	1.0		1.0	
	28	0.4	0.2-0.9	0.4	0.2-1.0
	>28	0.5	0.2-1.5	0.5	0.1-1.9
# of days of a period	2-4	1.0		1.0	
	5	1.0	0.5-1.9	1.8	0.8-3.9
	>5	1.2	0.5-2.7	2.2	0.8-5.9
Ever pregnant	No	1.0		1.0	
	Yes	0.4	0.1-1.6	1.0	0.2-5.4
# of pregnancies	1	1.0		1.0	
	2	0.5	0.2-1.4	0.6	0.2-1.9
	3	0.8	0.3-2.3	0.5	0.2-1.6
	>=4	0.5	0.2-1.4	0.6	0.2-1.9
Age at 1st pregnancy	13-17	1.0		1.0	
	18-19	0.8	0.3-1.8	0.4	0.1-1.1
	20-21	1.3	0.5-3.0	1.0	0.3-2.6
	22-23	1.0	0.3-3.4	1.3	0.4-4.7
	25-26	1.5	0.3-6.5	1.0	0.2-5.7
	>=27	0.5	0.1-1.6	1.9	0.6-5.7

Table 2 continued

Factor		ER- cases		ER+ cases	
		OR*	95%CI**	OR	95%CI
Use of birth control pills	No	1.0		1.0	
	Yes	0.8	0.5-1.6	0.9	0.4-1.8

* Adjusted for age, marital status, educational level, income and religion; ** 95% confidence interval.

Table 3. Odds ratio estimates of medical history for breast cancer by ER status, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1997

Factor		ER- cases		ER+ cases	
		OR*	95%CI**	OR	95%CI
Benign breast disease	No	1.0		1.0	
	Yes	1.5	0.8-2.9	0.7	0.3-1.5
History of other cancers	No	1.0		1.0	
	Yes	0.8	0.2-3.5	2.2	0.6-8.4
Radioactive therapy	No	1.0		1.0	
	Yes	1.8	0.4-7.5	2.4	0.5-11.9
Surgery on ovaries	No	1.0		1.0	
	Yes	1.0	0.5-1.8	1.1	0.5-2.2
Use of estrogen	No	1.0		1.0	
	Yes	0.8	0.4-1.5	1.0	0.5-2.3
Use of progesterone	No	1.0		1.0	
	Yes	1.0	0.4-2.6	1.2	0.4-3.6

* Adjusted for age, marital status, educational level, income and religion; ** 95% confidence interval.

Table 4. Odds ratio estimates of family history of breast cancer for the disease by ER status, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1997

Factor		ER- cases		ER+ cases	
		OR*	95%CI**	OR	95%CI
Breast cancer in 1st degree relatives	No	1.0		1.0	
	Yes	1.4	0.5-4.0	3.2	1.1-9.4
Breast cancer in 2nd degree relatives	No	1.0		1.0	
	Yes	3.1	1.3-7.3	6.2	2.4-15.7

* Adjusted for age, marital status, educational level, income and religion; ** 95% confidence interval.

Table 5. Odds ratio estimates of personal habits or behavior for breast cancer by ER status, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1997

Factor		ER- cases		ER+ cases	
		OR*	95%CI**	OR	95%CI
Ever smoking	No	1.0		1.0	
	Yes	0.9	0.5-1.6	0.8	0.4-1.6
Ever alcohol consumption	No	1.0		1.0	
	Yes	0.6	0.3-1.2	0.4	0.2-0.8
Use of electric blanket	No	1.0		1.0	
	Yes	1.4	0.7-2.8	0.8	0.4-1.8
Age at 1st sex	9-16	1.0		1.0	
	17-18	3.7	1.7-7.9	0.8	0.3-2.0
	19-20	2.2	0.9-5.8	1.8	0.7-5.0
	>=21	5.9	1.2-28.2	10.7	2.5-46.7
Age at 1st sex on a regular basis	9-16	1.0		1.0	
	17-18	3.4	1.1-11.1	2.5	0.7-9.2
	19-20	4.9	1.6-15.1	2.2	0.6-8.2
	21-22	3.7	1.1-13.1	3.0	0.8-11.3
	>=23	2.2	0.6-8.2	2.5	0.6-10.0

* Adjusted for age, marital status, educational level, income and religion; ** 95% confidence interval.

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Methyl-deficient diets, methylated ER genes and breast cancer: An hypothesized association

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Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5′ to the gene. Because CpG island methylation is an early event in carcinogenesis and because methyl-deficient diets could result in CpG island methylation, it is relevant to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes (as opposed to the disease with unmethylated ER genes). This molecular-based etiologic hypothesis may facilitate epidemiological research on the relationship between breast cancer and diet that has been unclear until now. *Cancer Causes and Control,* 1998, 9, 615-620

Key words: Breast cancer, estrogen receptors, gene, methyl-deficient diets, methylation.

Introduction

Breast cancer can be divided into two types according to the tumor estrogen receptor (ER) level: ER-positive or ER-negative. Because the presence or absence of ERs in breast cells may differentially affect the role some risk factors, such as estrogens, play on the etiology of the disease, it is reasonable to hypothesize that risk factor profiles of breast cancer vary by ER status of the disease. However, previous epidemiological studies on risk factors by ER status have obtained inconsistent results.[1-8] Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5′ to the gene.[9,10] We suggest that this observation may be critical in assessing breast cancer risk factors according to the ER status of the tumor.

The inconsistency in previous epidemiological studies by ER status may be related to problems in using total ER levels as an indicator of ER status without fully understanding the basis of ER level variation. Moreover, it is possible to misclassify an individual's ER status by just measuring total ER levels. For example, a tumor with a sparse distribution of ER-positive cells may be falsely considered ER-negative and a tumor with a dense distribution of ER-negative cells may be falsely considered ER-positive. This patchiness or variegation and failure to understand the underlying cause of ER level variation may have affected study results and conclusions.

Using the methylation status of the ER genes is less likely to be prone to the same effects of cellularity in defining ER status, and may help define a molecular-based etiologic hypothesis of breast cancer. Because CpG island methylation is an early event in carcinogenesis and may relate to breast cancer's lack of ER

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expression and because diets deficient in methyl-groups (such as methionine choline, and folate) can result in abnormal DNA methylation/carcinogenesis, it is reasonable to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes, but not for the disease with unmethylated ER genes.

CpG island methylation is an early event in carcinogenesis

CpG islands are located in the promoter regions of genes and their methylation status is important in gene transcription.[11-13] Active transcription requires an unmethylated state of 5′ sites that exist in normal adult tissues.[12,14,15] When CpG islands are methylated, chromatin structure can change, causing genes in these chromosome regions to become transcriptionally inactive.[16] These chromosome alterations may also result in DNA instability leading to tumorogenesis. In a study on colon cancer, Makos *et al.*[16] found that there is abnormal methylation of the CpG island areas on 17p in colon adenomas and the abnormality increases in colon cancers. Because allelic losses of chromosome 17p are characteristics of colon carcinomas, the results suggest that methylation precedes these allelic losses. Another study of colorectal tumors[14] showed that CpG island methylation of the ER gene increases with age in human colonic mucosa from normal individuals and can be found in all colorectal tumors. Vertino *et al.*[15] examined whether the aberrant methylation of CpG islands evolves as a function of immortalization and oncogene-induced neoplastic transformation of bronchial epithelial cells. They found that the methylation of CpG islands at 17p13 occurred during the immortalization of normal human bronchial cells and preceded oncogene-induced transformation. Because chromosome 17p13 deletions occur in lung tumorigenesis,[17] the results suggest that the methylation appears early in bronchial epithelial cell carcinogenesis that is related to immortalization.[15] In addition, Vertino *et al.*[13] found that aberrant CpG island methylation appeared during normal aging of fibroblasts and may predispose some cells to transform into cancer. Combined, these studies imply that CpG island methylation is an early event of carcinogenesis.

CpG island methylation of the ER gene may cause low ER expression in breast cancer

The human ER gene is located on chromosome 6q24-q27.[18,19] Recent studies have shown that ER-negative breast cancer is caused by a lack of ER gene transcription.[18,20] The lack of ER gene expression is related to methylation of the 5′ region of the gene:[21] 4 out of 5 samples were hypermethylated in ER-negative carcinomas and 13 of 15 were hypomethylated in ER-positive carcinomas.[20] Using human breast cancer cell lines, it was subsequently demonstrated that methylation of the CpG island in the 5′ region and first exon of the gene is responsible for lack of expression of ER gene in ER-negative breast tumors.[9] This was confirmed by reactivating the ER gene using inhibitors of DNA methylation, which demethylate the ER CpG island.[10] Although the results based on breast cancer specimens are more complex due to the heterogeneity of cell populations within a tumor, it was found recently that ER-negative tumors have higher mean scores of ER CpG island methylation than ER-positive tumors.[22] By analogy to the colorectal cancer story given above, ER gene methylation may be an early event in some breast cancer (*i.e.* ER negative), if breast cancer shares similar molecular mechanisms to other tumors.

Risk factors may differ depending upon the methylation status of the ER genes

Certain risk factors may be important for tumors with methylated genes and other factors may be significant for other tumors. For example, in a recent study,[23] it was found that lung cancers from smokers and from animals exposed to tobacco-specific carcinogens had a low incidence of CpG island methylation of the ER genes, while lung cancers of non-smokers and spontaneous tumors in animals had a high incidence of methylation. For breast cancer, it can be postulated that factors that can cause or facilitate CpG island methylation of the ER gene may only increase the risk of breast cancer with ER gene methylation. Due to a lack of receptors resulting from the methylation, breast cells with methylated CpG islands may not be affected by subsequent exposures to estrogens during their transformation into cancer cells. On the contrary, tumors with unmethylated ER genes, and therefore with receptors, may be more susceptible to factors that can interact with ERs. If these differences exist, breast cancers with and without ER gene methylation will have distinct risk factor profiles.

Methyl-deficient diets could result in abnormal DNA methylation and therefore are more likely to be related to breast cancer where the ER gene CpG islands are methylated

No studies have been conducted on breast cancer risk factors according to the methylation status of the ER gene. However, the possibilities discussed above imply an association of methyl-deficient diets with breast cancers where the ER gene is methylated. Such an

association, if it exists, may be based on the following hypothesized mechanisms. It is suggested that diets deficient in methyl-groups (such as methionine and folate) or high in methyl group antagonists (such as alcohol) cause increased DNA methyltransferase (DNA-MTase) activity.[24] There may be two types of DNA-MTase activities: *de novo* methyltransferase activity and maintenance methyltransferase activity.[25] Elevated *de novo* DNA-MTase activity may initiate[25,26] and elevated maintenance DNA-MTase activity may subsequently spread and maintain[26] methylation of the usually unmethylated CpG sites, possibly through the disruption of the boundaries that normally protect CpG islands from methylation.[26] Methylation of the CpG sites after a relatively long-term methyl-deficient diet has been directly demonstrated during the transition to tumor in animals,[27] although it was not suggested in a study in humans,[28] in which short-term dietary methyl group restriction was used and methylated urine metabolites rather than methylation of the CpG sites was measured. The hypermethylation of the CpG islands silences tumor suppressor genes[29] such as the ER gene[30] and therefore is related to the occurrence of cancer. Methyl-deficient diets can also lower cellular levels of the methyl donor S-adenosylmethionine.[31-33] Reduced S-adenosylmethionine can cause global genomic hypomethylation[25,32,34,35] and therefore the activation of some oncogenes.[26] Decreased S-adenosylmethionine can also facilitate the activity of DNA-MTase as a mutator enzyme, leading to CpG mutagenesis.[25] Probably as a result of these DNA changes, diets reducing methyl-group availability may increase the risk of cancer. Observations in animal models[36] and humans[24,37,38] support this. In Giovannucci *et al.*'s study,[24] a combination of high alcohol and low methionine and folate intake conferred a relative risk of 7.4 for distal colon cancer. Because low dietary methyl-components may cause (1) the methylation of ER gene CpG islands that reduces tumor suppressing activities of the ER genes, (2) global genomic hypomethylation that may activate some oncogenes and (3) CpG mutagenesis, it is reasonable to hypothesize that methyl-deficient diets and those high in methyl-antagonists are likely to be related to breast cancer primarily with methylated ER genes. Figure 1 depicts the hypothesized association.

Our hypothesis of the association between methyl-deficient diets and breast cancer with ER gene methylation suggests the need to study breast cancer risk factors with respect to specific molecular characteristics. Tumors with and without a specific molecular characteristic may have different causal pathways and therefore have different risk-factor profiles. The differences may originate from two things. First, the change in methylation pattern is probably not inherited. Rather, it may result from a number of environmental or somatic factors that do not co-occur in cancers without this molecular change. Second, even though methylation changes exist (due to either environmental exposures or somatic factors), they may not cause cancer alone. It is likely that methylation imparts susceptability to cells and causes cancer in the presence of other genetic or environmental factor(s). Because these other factors have their effects in conjunction with this susceptibility, their association with cancer would be different, depending upon whether a tumor has such susceptibility.

Several issues should be considered in the exploration of the relationship between methyl-deficient diets, breast cancer and methylation status of the ER gene. First, methyl-deficient diets are also associated with global genomic hypomethylation related to the occurrence of cancer. If the hypomethylation could occur without methylation of CpG islands, breast cancer without methylated ER genes may also be susceptible to the effects of methyl-deficient diets. While we do not exclude this possibility, it is unlikely because widespread genomic hypomethylation and methylation of CpG islands usually exist simultaneously in tumor cells.[26] Second, the metabolism of methyl groups is influenced by methylenetetrahydrofolate reductase (MTHFR).[39,40] A mutation in the MTHFR gene, which is common in many populations,[41] can reduce specific MTHFR activity, leading to decreased methionine and S-adenosylmethionine levels.[42] Decreased S-adenosylmethionine in individuals with the MTHFR mutation appears only in the presence of low folate status.[43] Therefore, the association between methyl-deficient diets or methyl-antagonists and cancer might be stronger among people with mutated MTHFR genes, as suggested by recent studies.[44,45] The effect of methyl-deficient diets on breast cancer with methylated ER genes, if any, may be modified by the MTHFR genotype, which should be considered in studies on the hypothesized association. Finally, the hypothesized association between methyl-deficient diets and the risk of breast cancer with methylated ER gene is based on the hypothesis that breast cancers with and without methylated ER genes are two different entities that may have different etiologic pathways. This hypothesis is tenable because the methylation status of CpG islands has been suggested as an early event in the development of cancer. However, if methylated CpG islands also occur as a function of tumor progression after a tumor develops, they may appear in some late-stage breast cancers that were unmethylated at their early stage, leading to the misclassification of real methylation status. Early-stage tumors should be used if this is true.

Many epidemiologic studies of cancer risk factors have not distinguished tumors by genetic or epigenetic

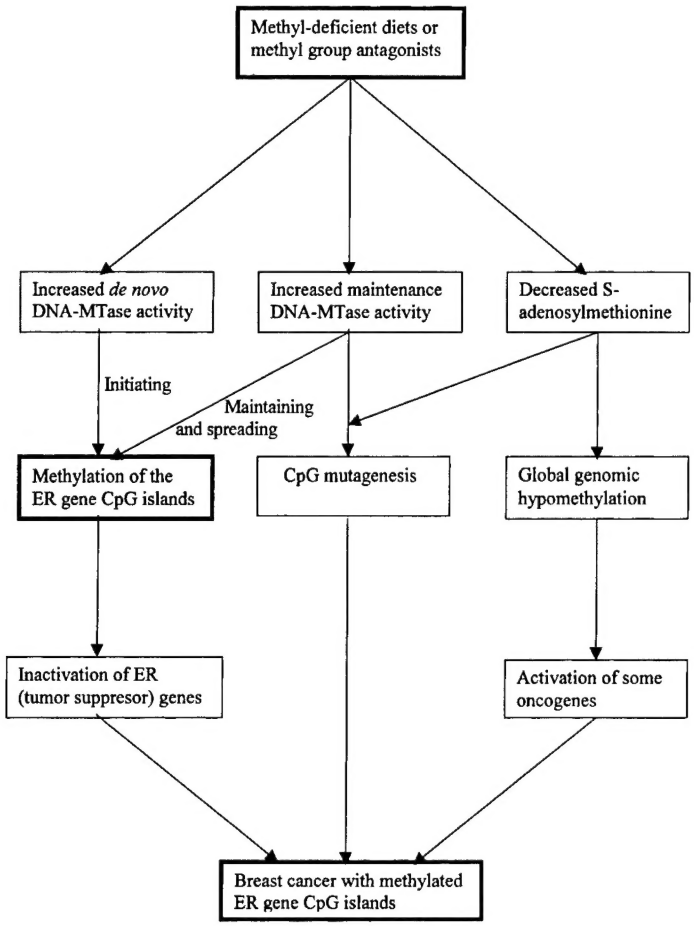

Figure 1. Hypothesized association between methyl deficient diets and breast cancer with methylated ER gene CpG islands.

characteristics.[46] The pooling of similar cancers having different causal pathways would dilute the ability to detect risk factors for each pathway. Without information on the methylation status of ER gene CpG islands, previous epidemiological studies on fruits/vegetables (rich in folate[24,37]) and poultry/fish/dairy products (rich in methionine[24]) have found either an association between the lack of these dietary factors and increased risk of breast cancer,[47-51] or no association.[52,53] Studies on alcohol consumption (a methyl group antagonist) also have showed a null or weak positive association with breast cancer.[54-56] Because methyl-deficient diets and methyl group antagonists are related to abnormal DNA methylation, they may be a risk factor for tumors with methylated ER genes, but not for those without. The lumping of tumors with different ER gene methylation statuses may have led to an estimate of a diluted association. Case-control studies on methyl-deficient diets, in which breast cancers are distinguished by the methylation status of the ER genes, can be used to explore such a possibility. Cohort studies are also feasible by examining the ER methylation status of tumors among women with and without methyl-deficient diets. Studies that distinguish different genetic or epigenetic status of tumors would improve research on the relationship between risk factors and the disease,[57,58] increasing our ability to comprehend diet-breast cancer relationships that have not been clear to this point.

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